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SHORT REPORT

Emergence of ST15 *Klebsiella pneumoniae* Clinical Isolates Producing Plasmids-Mediated RmtF and OXA-232 in China

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Background: RmtF, as 16S rRNA methyltransferase, leads to high-level resistance to aminoglycoside and is now barely reported.

Methods and Results: Three *rmtF*-positive *Klebsiella pneumoniae* isolates, belonging to the pandemic clone sequence type 15, were isolated from children and coproduced $bla_{\text{OXA-232}}$ and $bla_{\text{CTX-M-15}}$. The *rmtF* gene was located on an IncFIB transformable plasmid of 128,536-bp and $bla_{\text{OXA-232}}$ was on a 6141-bp ColKP3 plasmid, respectively.

Conclusion: Plasmids with *rmtF* found worldwide, shared relatively low similarity, and merely matched partly in their multidrug resistance region. Notably, clinical isolates coproducing *rmtF* and $bla_{\text{OXA-232}}$ are gradually increasing in China.

Keywords: *Klebsiella pneumoniae*, *RmtF*, $bla_{\text{OXA-232}}$, ST15, aminoglycoside

Introduction

Carbapenemase-producing *Klebsiella pneumoniae* have become a great challenge for antimicrobial chemotherapy, while aminoglycosides can lower the mortality rate effectively in combination therapy of them.[1] 16S rRNA methyltransferase (16S-RMTase), which induces high-level resistance to aminoglycosides, is now commonly encountered in *Enterobacterales* worldwide. In China, 16S-RMTases have been found in *Enterobacterales* from both humans and animals with similar isolation rates, among them *rmtB* was the most common followed by *arm*A.[2] RmtF was a 16S-RMTase firstly identified in France in 2012,[3] and thereafter only happened individually in countries like the United States, India and some European countries.[4–6]

Methods and Results

Recently, three *Klebsiella pneumoniae* strains K60, K65 and K77 producing RmtF coupled with OXA-232 were isolated from neonates in Shanghai, China. Ethics committee approval was obtained from the institutional review board of Huashan hospital for these isolates, and verbal informed consent from patients' parents was also accepted and approved by Huashan Hospital.

As determined by the reference Clinical and Laboratory Standards Institute (CLSI) broth microdilution method,[7] all three *K. pneumoniae* isolates were highly resistant to aminoglycosides and most antimicrobial agents tested, except carbapenems, to which the resistance produced in low degree (Table 1). The consistency of

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Table 1 Minimal Inhibitory Concentrations (MICs) of *K. Pneumoniae* K60, K65, K77 and Their *rmtF*-Positive Escherichia Coli Transconjugants

Antimicrobials	MIC (μg/mL) for:						
	Recipient	Donors			Transconjugants		
	J53AziR	K60	K65	K77	J60	J65	J77
Amikacin	≤1	>128	>128	>128	>128	>128	>128
Ertapenem	≤0.25	8	8	16	≤0.25	≤0.25	≤0.25
Imipenem	0.25	1	1	1	0.25	0.25	0.25
Meropenem	≤0.03	4	4	4	≤0.03	≤0.03	≤0.03
Cefmetazole	2	16	16	32	2	2	2
Cefazolin	4	>32	>32	>32	>32	>32	>32
Cefuroxime	16	>64	>64	>64	>64	>64	>64
Ceftriaxone	≤0.25	>32	>32	>32	>32	>32	>32
Ceftazidime	0.5	>32	>32	>32	32	32	32
Cefepime	≤0.06	>128	>128	>128	32	32	32
Ceftazidime/avibactam	0.25	0.5	0.5	0.5	0.25	0.25	0.5
Cefoperazone/sulbactam	≤1	>128	>128	>128	32	16	16
Tigecycline	0.25	2	2	2	0.25	0.25	0.25
Polymyxin B	0.25	0.5	0.5	0.5	0.5	0.25	0.25
Aztreonam	≤1	>128	>128	>128	64	64	64
Ciprofloxacin	≤0.06	>8	>8	>8	0.25	0.25	0.5
Levofloxacin	≤0.125	>16	>16	>16	0.5	0.5	0.5
Trimethoprim/sulfamethoxazole	≤0.25	>32	>32	>32	>32	>32	>32

the pulsed-field gel electrophoresis (PFGE) image of strain K60, K65 and K77 indicated that they were identical (Figure 1).

Multiplex PCRs were performed to detect Ambler class A, B and D β-lactamase-encoding genes and 16S rRNA methyltransferase-encoding genes[6,8,9] and followed by DNA sequencing. *RmtF* and $bla_{OXA-232}$ genes were positive for all three *K. pneumoniae* isolates. A cloning experiment was then performed. *E. coli* DH5α was transformed with these plasmids, which yielded the vector pBad33 with a 1.5-kb insert containing *rmt*F,[3] and then their resistance to aminoglycosides transferred from sensitive to highly resistant (Table 1).

Mating-out assays were performed to establish the transferability of the *rmtF* using the azide-resistant *E. coli* J53 as recipient strain (selected with gentamicin at 25 mg/L and sodium azide at 150mg/L). Transconjugants were highly resistant to aminoglycosides, cephalosporins and trimethoprim/sulfamethoxazole, while the plasmid containing $bla_{OXA-232}$ was not obtained.

Genomic DNA of *K. pneumoniae* K60, K65 and K77 were subjected to whole-genome sequencing (WGS) through Illumina (Illumina, San Diego, CA, USA) short-read sequencing and Nanopore (Oxford, UK) long-read sequencing. Both Genome and plasmids of these strains showed substantial homology, only with the difference of single-nucleotide polymorphism (SNP) level. The genome of these strains was ca. 5335-kb in length and was mapped to CP008929.1 (Nepal), CP015990.1(China), CP022127.1(United States), with the proportion over 98% of each, suggesting the possibility of widespread. MLST showed that it belonged to the pandemic clone sequence type 15 (ST15) (https://cge.cbs.dtu.dk/services/MLST/), which is one of the dominant global type, associated with a range of beta-lactamases, including OXA,[10] NDM[11] and CTX-M.[12]

Each strain contained three plasmids carried resistance genes. P1 was a 136,315-bp IncFII plasmid harboring *aph (3")-Ib, aph (6)-Id, qnrB1,* $bla_{CTX-M-15}$, bla_{TEM-1}, *dfrA14* and *sul2*, causing resistance to aminoglycosides, quinolones, beta-lactams and trimethoprim/sulfamethoxazole. P2 was a 128,536-bp IncFIB plasmid harboring *aac (6')-Ib, rmtF, arr-2* and *cat*, causing resistance to aminoglycosides, rifampicin, chloramphenicol. The $bla_{OXA-232}$ was carried on P3, a 6141-bp ColKP3 nonconjugative plasmid identical to that identified previously,[13] and was now widely reported in the world.[14,15] In China, such plasmid with $bla_{OXA-232}$ first emerged in 2017,[16] and further appeared in the clonal dissemination of ST15 carbapenem-resistant *K. pneumoniae*

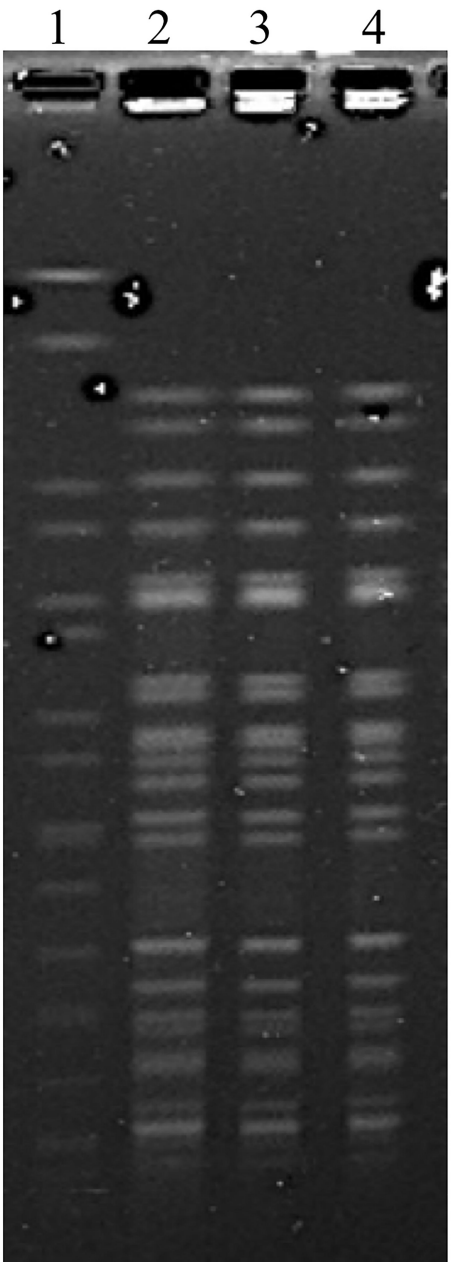

Figure 1 PFGE of *K. pneumoniae* K60, K65 and K77. Lanes 1, marker *Salmonella braenderup* H9812; line 2 to 4, PFGE image of *K. pneumoniae* K60, K65 and K77.

among elderly patients.[17] Notably, in this study, $bla_{\text{CTX-M-15}}$ and *rmtF* transmitted by plasmid P1 and P2 in the conjugation experiment, while $bla_{\text{OXA-232}}$ on P3 could not cotransfer with them. This result was completely consistent with previous reports, but in which the *rmtF* and $bla_{\text{CTX-M-15}}$ genes were located on the same 160 kb plasmid.[6] Among them, plasmid P2 with *rmtF* was barely reported in China.

P2 was perfectly mapped to pPMK1-B (GenBank accession no. CP008931.1),[14] except a multidrug resistance region (MRR) of 16,839-bp carrying all the resistance genes of P2 (Figure 2). The MRR was flanked by genes of Tn3 family transposases on both sides, and also contained *IS91* and *IS6100*. Mobile elements like these can cluster and be combined with resistance genes, bringing about multiple resistance transfer of plasmids.[18] Genes encoding YafQ family toxin proteins were also found in the MRR, such toxin-antitoxin proteins were frequently located on plasmids where they serve to promote plasmid's stability and maintenance in the bacterial host.

In all, 17 full sequences of plasmids with *rmtF* were found in GenBank data, which were reported worldwide, and then they were compared to P2 using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Except for pSg1-NDM (GenBank accession no. CP008931.1), an IncR plasmid found in Singapore, the rest of these plasmids were IncF plasmids with only one MRR. As one of the most frequent plasmid types, plasmids of the IncF group have a primary role in the antimicrobial resistance of *Enterobacterales* and show rapid evolution.[19] However, these plasmids shared relatively low similarity, merely matched imperfectly in their MRR, while all of these MRR were jointed to mobile elements like Tn3 family transposases (Figure 2).

Conclusion

Worryingly, the co-occurrence of 16S rRNA methyltransferases and carbapenemases has been increasingly reported among *K. pneumoniae* in recent years.[20] *Enterobacterales* isolates producing *rmtF* used to be extremely rare in China, but in recent years relevant reports have emerged and always accompanied with coproduction of OXA-232.[21,22] In countries like India and the UK, the detection rate of *rmtF* increased rapidly, suggesting the possibility of its speedy spread. Plasmids with *rmtF* gene often featured resistance genes acquiring through mobile elements and plasmid addiction modules made up of toxin-antitoxin proteins, which led to the stable persistence of clinical isolates and ultimately resulted in multidrug resistance to almost all of the clinically available antibiotics. In this study, all three clinical isolates were susceptible only to cefepime/zidebactam, ceftazidime/avibactam, tigecycline, polymyxin B and imipenem. This made clinical choices extremely limited if economic cost and medicine availability were taken into consideration. This represented the worry toward the distribution and transmission of clinical Isolates with analogous plasmids as a global public threat. To screen these resistance genes and comprehend their transmissibility in time, plasmid analysis may be a useful supplementary method for medical institutions. Based on correlative results, measures like contact

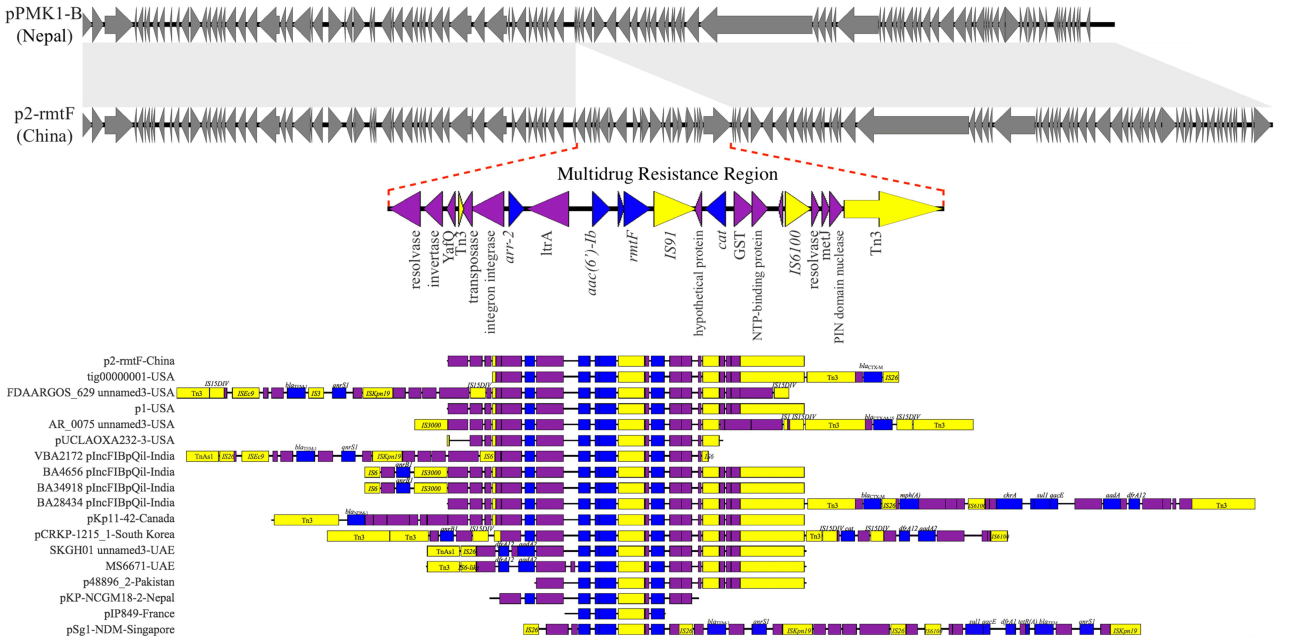

Figure 2 Major structural features of P2, pPMK1-B and the MRR of other plasmids with *rmtF* gene. Plasmids sequenced analyzed were compared as follows: P2 was compared with pPMK1-B, the MRR of P2 was compared with the MRR of other plasmids carrying *rmtF* gene: tig00000001-USA (121,057-bp, NZ_CP021758.1), FDAARGOS_629 unnamed3-USA (138,560-bp, CP044045.1), p1-USA (142,764-bp, CP033947.1), AR_0075 unnamed3-USA (93,870-bp, CP032188.1), pUCLAOXA232-3-USA (83,730-bp, CP012569.1), VBA2172 pIncFIBpQil-India (127,300-bp, CP036321.1), BA4656 pIncFIBpQil-India (164,210-bp, CP035907.1), BA34918 pIncFIBpQil-India (164,195-bp, CP036193.1), BA28434 pIncFIBpQil-India (130,775-bp, CP036328.1), pKp11-42-Canada (146,695-bp, KF295829.1), pCRKP-1215_1-South Korea (130,922-bp, CP024839.1), SKGH01 unnamed 3-United Arab Emirates (84,941-bp, NZ_CP015503.1), MS6671-United Arab Emirates (84,940-bp, LN824138.1), p48896_2-Pakistan (114,815-bp, CP024431.1), pKP-NCGM18-2-Nepal (9812-bp, AB824739.1), pIP849-France (4710-bp, JQ808129.1), pSg1-NDM-Singapore (90,103-bp, NZ_CP011839.1). Grey symbols indicate identical plasmid regions of P2 and pPMK1-B, while gray shading indicates >99% identity of them, and the red dotted line indicates their different component. Resistance genes are indicated by blue symbols. Transposon-related genes the class 1 integrase gene, and insertion sequences are indicated by yellow symbols. Other genes are indicated by violet symbols. Labels common to these MRRs appear only in the P2 diagram.

isolation and environmental cleaning can also be performed to avoid nosocomial outbreak.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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